ELF4 is required for oscillatory properties of the circadian clock

ELF4 Is Required for Oscillatory Properties of the Circadian Clock[1][W]

Harriet G. McWatters[2], Elsebeth Kolmos[2], Anthony Hall, Mark R. Doyle, Richard M. Amasino, Péter Gyula, Ferenc Nagy, Andrew J. Millar, and Seth J. Davis*

Department of Plant Sciences, University of Oxford, Oxford OX1 3RB, United Kingdom (H.G.M.); Max Planck Institute for Plant Breeding Research, D–50829 Cologne, Germany (E.K., S.J.D.); School of Biological Sciences, University of Liverpool, Liverpool L69 7ZB, United Kingdom (A.H.); Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706 (M.R.D., R.M.A.); Institute of Plant Biology, Biological Research Centre of the Hungarian Academy of Sciences, H–6726 Szeged, Hungary (P.G., F.N.); and Institute of Molecular Plant Sciences, University of Edinburgh, Edinburgh EH9 3JH, United Kingdom (A.J.M.)

Circadian clocks are required to coordinate metabolism and physiology with daily changes in the environment. Such clocks have several distinctive features, including a free-running rhythm of approximately 24 h and the ability to entrain to both light or temperature cycles (zeitgebers). We have previously characterized the *EARLY FLOWERING4* (*ELF4*) locus of Arabidopsis (*Arabidopsis thaliana*) as being important for robust rhythms. Here, it is shown that *ELF4* is necessary for at least two core clock functions: entrainment to an environmental cycle and rhythm sustainability under constant conditions. We show that *elf4* demonstrates clock input defects in light responsiveness and in circadian gating. Rhythmicity in *elf4* could be driven by an environmental cycle, but an increased sensitivity to light means the circadian system of *elf4* plants does not entrain normally. Expression of putative core clock genes and outputs were characterized in various *ELF4* backgrounds to establish the molecular network of action. *ELF4* was found to be intimately associated with the *CIRCADIAN CLOCK-ASSOCIATED1* (*CCA1*)/*LONG ELONGATED HYPOCOTYL* (*LHY*)-*TIMING OF CAB EXPRESSION1* (*TOC1*) feedback loop because, under free run, *ELF4* is required to regulate the expression of *CCA1* and *TOC1* and, further, *elf4* is locked in the evening phase of this feedback loop. *ELF4*, therefore, can be considered a component of the central *CCA1/LHY-TOC1* feedback loop in the plant circadian clock.

Many organisms have evolved circadian clocks to facilitate optimal timing of rhythmic behaviors. Plants use an endogenous oscillator and predictable signals from the environment to anticipate changes in circadian time. Key outputs controlled by the clock include the timing of germination, optimization of photosynthetic processes relative to the time of day, and floral transition. Each of these has been shown to be crucial for plant fitness (Green et al., 2002; Dodd et al., 2005). In recent years, several molecular components associated with the plant clock have been identified. Most of these components are themselves circadian regulated, with peak expression of each phased to occur at a specific time of day. For example, the MYB-related transcription factors, *CIRCADIAN CLOCK-ASSOCIATED1* (*CCA1*) and *LONG ELONGATED HYPOCOTYL* (*LHY*; Schaffer et al., 1998; Wang and Tobin, 1998), are morning-specific genes, both acting in a feedback loop on the pseudoresponse regulator *TIMING OF CAB EXPRESSION1* (*TOC1*), which peaks in the evening (Strayer et al., 2000; Alabadi et al., 2001; Mas et al., 2003). This transcription-translation feedback loop has been placed at the core of the Arabidopsis (*Arabidopsis thaliana*) clock (Alabadi et al., 2001). The original single-loop model was recently extended to incorporate additional loops (Farre et al., 2005; Locke et al., 2005, 2006; Salome and McClung, 2005a; Zeilinger et al., 2006). Beyond this core, the wider plant circadian system constitutes a complex network of multiple and interconnected pathways, many of which feed back on each other, controlling responses to light, temperature, and day length. These features are poorly understood.

Previously, we identified *early flowering4* (*elf4*) from Arabidopsis and showed that *ELF4* is important for circadian precision and normal clock function (Doyle et al., 2002). The *elf4* loss-of-function mutation attenuated

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[2] These authors contributed equally to the article.

* Corresponding author; e-mail davis@mpiz-koeln.mpg.de; fax 49–(0)221–5062267.

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free-running rhythmicity in all clock outputs tested and this included components believed to make up the central clock machinery (Doyle et al., 2002; Kikis et al., 2005; this article). Circadian specificity of *ELF4* within the clock was only partially defined with these studies.

Light signals perceived by photoreceptors, including phytochromes and cryptochromes (Lin, 2002; Nagy and Schafer, 2002; Quail, 2002), are the most important environmental inputs to the plant circadian clock (Ni, 2005). Photoperception allows entrainment of the clock to dawn and dusk cues, allowing correct phasing of the various clock-controlled genes and pathways (Salome and McClung, 2005b). Clock control of light-signaling pathways is critical for photoperiodic regulation of many aspects of Arabidopsis development, including hypocotyl elongation and seasonal induction of the floral transition. Here, *ELF4* has been implicated in phytochrome B signaling because *elf4* seedlings are hyposensitive to red light and *ELF4* mRNA levels are low in the *phyB* mutant. Further, it has been interpreted that *ELF4* controls red-light repression of hypocotyl elongation (Khanna et al., 2003) and that *ELF4*, together with *TOC1*, plays a major role in phytochrome-mediated input to the clock (Kikis et al., 2005). The early flowering behavior of *elf4* is accompanied by misregulation of the flowering activator *CONSTANS* (*CO*), implying *ELF4* acts on flowering time by regulating expression of *CO* (Doyle et al., 2002). Connecting *ELF4*'s action on the clock to downstream red-light perception is required to understand the pleiotropic nature of the *elf4* mutations.

We have shown previously that *ELF4* is expressed in the evening and that the *elf4* loss-of-function mutant has low *CCA1* expression leading to arrest of the *elf4* oscillator after one cycle under free run (Doyle et al., 2002). Recently, it was shown that *elf4* also has low *LHY* transcript levels, implicating *ELF4* in a feedback loop with *CCA1* and *LHY* (Kikis et al., 2005). Here, we expand the understanding of *ELF4* function in the circadian clock network. We found that the ability to reentrain to a light-dark (LD) zeitgeber was altered in *elf4* mutant plants. In addition, *elf4* seedlings released into continuous light (LL) exhibited an immediate attenuation of rhythmicity. Plants overexpressing *ELF4* were modestly late flowering and had a long circadian period. Furthermore, we confirmed the hypothesis that *ELF4* acts on the *CCA1/LHY-TOC1* feedback loop via detailed molecular expression analysis of core clock genes in informative *ELF4* genotypes. Finally, although the Arabidopsis clock robustly entrains to ambient temperature cycles (Somers et al., 1998; Michael and McClung, 2002; Michael et al., 2003), *elf4* mutants did not properly free run after exposure to temperature cycles.

RESULTS

Hypo- and Hypermorphic Red-Light Signaling in *elf4* Plants

Under natural 24-h days, the LD rhythm defines the diurnal environment. However, signaling through light input pathways in plants is itself a clock-controlled process, being gated by so-called zeitnehmer functions, one of which requires *ELF3* (McWatters et al., 2000). Previous reports on *ELF4* characterization have supported *ELF4* action in a phytochrome B-dependent pathway of red-light perception (Khanna et al., 2003). Accordingly, we tested *elf4-1* mutants and *ELF4* overexpression (*ELF4-ox*) lines for alterations in detecting light input signals and/or diurnal processing of information (*ELF4-ox* construction is described below). *elf4-1* seedlings had a mild hypocotyl elongation phenotype under a range of fluences of red light because *elf4-1* appeared hyposensitive to red-light repression of elongation growth (Fig. 1A). We thus confirm previous work by the Quail group (Khanna et al., 2003). Interestingly, *ELF4-ox* lines were indistinguishable from wild type under these assay conditions. Thus, if *ELF4* is a component of proper red-light perception, then it is not a genetically limiting factor for the repression of hypocotyls by light.

A gating assay was conducted to test whether the red-light defects in *elf4-1* were in part due to alterations in circadian processing of light information. For this, wild-type and *elf4-1* plants harboring the *CAB2*:*luciferase* (*LUC*) marker were entrained to 12/12 LD cycles and replicate samples were placed into continuous darkness (DD). From subjective dark (zeitgeber time [ZT] = 12, noted here as at the start of the Fig. 1B graph; transfer time = 0) at 2-h intervals, a set of replicate samples was given a 5-min pulse of red light and the acute response of light activation of *CAB2*:*LUC* induction was assayed. As reported previously for white-light pulses (McWatters et al., 2000), we could confirm that wild-type Arabidopsis has a gated response of *CAB2* induction (Fig. 1B). The response to light was at a maximum during the early part of the subjective day, around the time when the plants anticipated the transition of dark to light (subjective dawn; time 12 h in Fig. 1B), indicating the gate is open during the (subjective) day but closed during the (subjective) night. The *elf4-1* mutant showed defects in its response to red-light treatments over the course of the entire experiment, but most especially during subjective night (times 0–12 h and 24–36 h in Fig. 1B). In *elf4-1* mutants, the gate was open during subjective night (Fig. 1B) when *elf4-1* displayed high activation of *CAB2* in response to the light pulse. These plants have increased sensitivity to light at night relative to wild type and thus *elf4* is a partial gating mutant. Red-light perception in the *elf4-1* mutant is thus altered, at least in part, because of an underlying clock defect that affects the gating of this red-light response pathway.

elf4-1 Mutants Arrest Their Clock in the Evening

It was noted that, after transfer to constant conditions following exposure to LD cycles, *elf4-1* mutant plants displayed weak rhythmicity on the first day (Doyle et al., 2002). This could mean that the oscillator was, upon transfer to constant conditions, running

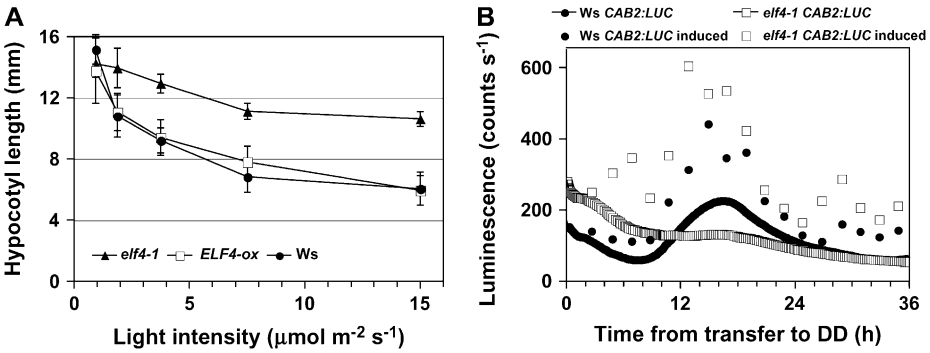

Figure 1. *ELF4* is involved in red-light response and acts at night. A, Hypocotyl length of 1-week-old seedlings grown under continuous red light. *elf4-1* has a long hypocotyl under a range of red-light fluences where *ELF4-ox* has no phenotype. B, *ELF4* gates light input to the clock during the night (ZT13 to ZT23), here shown as the difference in *elf4-1 CAB2:LUC* luminescence in light-induced versus noninduced seedlings. Seedlings were entrained in 12/12 LD cycles and transferred to DD at dusk (ZT12). Time in hours since the start of transfer (hence time = 0) was ZT12. The experiment was repeated twice.

down rather than stopping instantly. To understand the kinetics of the *elf4* oscillator, we undertook an assay of oscillator behavior following the transfer from entraining conditions to DD. Seedlings harboring the *CAB2:LUC* reporter were entrained to 8/16 LD cycles and then transferred to DD at dusk (ZT8). At 3-h intervals from 1 h after the light-to-dark transition, a 5-min red-light pulse was given to replicate plates of seedlings and luminescence was measured over the next 48 h. This light pulse is not sufficient to reset the clock in wild type (Millar et al., 1992; McWatters et al., 2000; Covington et al., 2001; Hall et al., 2002), but it does induce a circadian peak of *CAB2* activity, the timing of which is under circadian control in wild-type plants (Millar and Kay, 1996).

Until 32 h after the last dawn (i.e. subjective dusk for these plants previously entrained to 8/16 LD), the timing of the peak in *elf4* seedlings was indistinguishable from that of wild-type plants (Fig. 2A). However, the two sets of seedlings responded differently to pulses given at or 36 h after the last dawn (*t* test; $P < 0.05$): Wild-type seedlings continued to show circadian control, but the peak of *CAB2:LUC* in *elf4-1* occurred about 30 h after the pulse, regardless of when the pulse was given (Fig. 2A). Thus, the circadian clock in *elf4* runs down at the end of the first subjective day in DD to a point where it is strongly reset by even a brief light pulse. We interpret this as although rhythmicity can be driven by a light zeitgeber in *elf4*, *ELF4* is needed to sustain clock activity beyond the end of the first subjective day in DD.

Characterization of *ELF4-ox* Plants

We previously concluded based on loss-of-function studies that *ELF4* is both a repressor of the floral transition and required to sustain normal clock function (Doyle et al., 2002). Because *ELF4* expression is normally rhythmic, plants overexpressing *ELF4* (*ELF4-ox*) under the control of the constitutive cauliflower mosaic virus 35S promoter (Supplemental Fig. S1A) were tested to see whether rhythmicity of transcription was required for *ELF4* function. We confirmed that *elf4-1* was partially insensitive to photoperiod (early flowering in long days [*t* test; $P < 0.01$] and short days [*t* test; $P < 0.001$]). In contrast, *ELF4-ox* lines were only late flowering under inductive (long-day) photoperiods (*t* test; $P < 0.001$). Under the noninductive conditions of short days, *ELF4-ox* plants showed no additional delay in flowering (*t* test; $P = 0.21$; Fig. 3, A and B). This finding confirms that *ELF4* is a floral repressor that works to coordinate the floral transition as part of the photoperiod pathway.

Because *elf4-1* is a severe clock mutant under light or in darkness, it was reasoned that *ELF4-ox* lines should also show circadian alterations. Three independent transgenic lines were tested for alterations in circadian leaf movement rhythms. All lines showed an increased free-running period under LL (Table I; Supplemental Fig. S1). These results were confirmed for molecular rhythms of *ELF4-ox* plants harboring the morning *CAB2:LUC* and the evening *CCR2:LUC* reporters (Table I; Fig. 3, C–F). These lines also had rhythms with longer periods under LL after entrainment to LD cycles (Fig. 3, C and D). In darkness, *ELF4-ox* peaked later than wild type most significantly for the evening marker *CCR2:LUC* (Fig. 3, E and F). Thus, *ELF4* modulates rhythmicity of multiple clock outputs. Here, we define based on these misexpression studies that *ELF4* is a strong genetic repressor of clock periodicity.

Entrainment to LD Cycles Is Altered in *elf4-1* Mutants

The gating assay (Fig. 1B) showed us that *elf4-1* plants display greater sensitivity to light than wild type. *CCA1* and *CAB2* are both under clock control and normally rise during the late night with peak at or shortly after dawn, respectively. They are also regulated directly by light. *CCR2* expression is also clock controlled, but is less directly affected by light (Suarez-Lopez et al., 2001; Kim et al., 2003), unlike *CAB2* or *CCA1*. We measured *CCA1*, *CAB2*, and *CCR2* expression

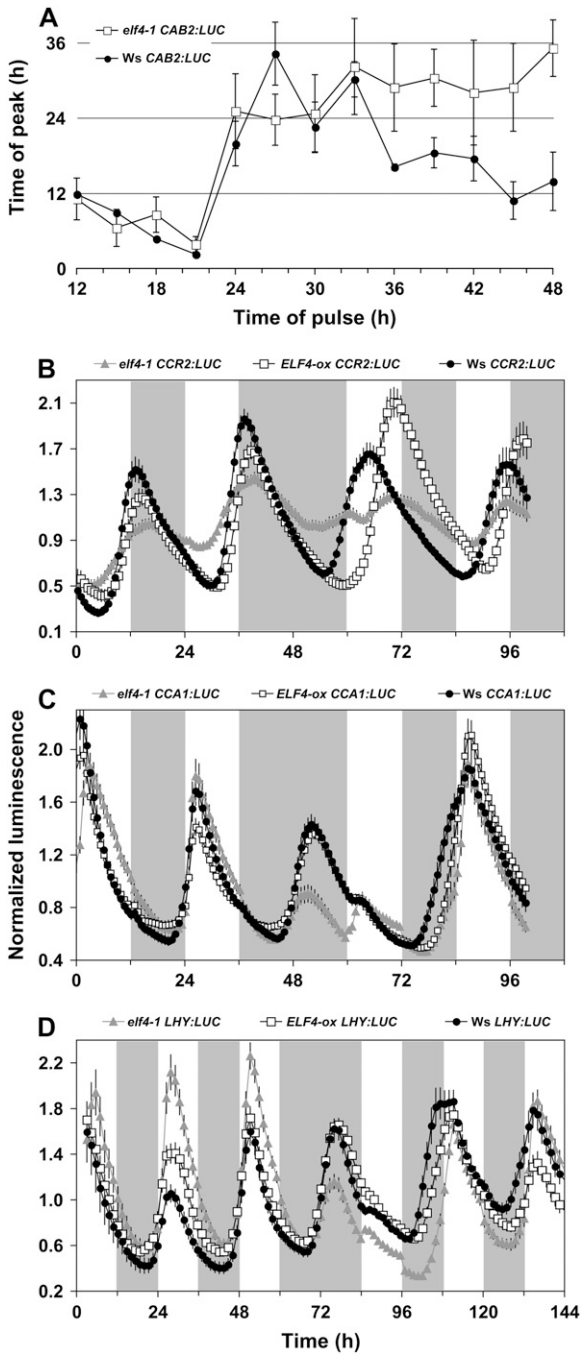

Figure 2. The *elf4* clock runs for 1 d and stops at subjective dusk (approximately time 32 h). A, Time to peak of *CAB2*:*LUC* activity in dark-adapted *elf4-1* seedlings after red-light pulse treatment. Seedlings were entrained in 8/16 LD cycles and then transferred to darkness at dusk (ZT8). Time of pulse is shown as hours since last dawn; 5 min of red light were given at 3-h intervals from time 9. Error bars represent SEM. The experiment was repeated twice. B to D, Normalized *CCR2*:*LUC*, *CCA1*:*LUC*, and *CCR2*:*LUC* profiles of *elf4-1* and *ELF4-ox* seedlings, compared to the wild type, before and after exposure to jet lag (an extended night of 24 h long) under LD cycles. White bars indicate light intervals and gray bars indicate darkness.

via *LUC* reporter activity in long- and short-day LD cycles to compare the effects of clock and light control on these genes.

In *elf4-1* under long or short days, there was a strong reduction in the rising of gene expression during darkness and, instead, there was an abrupt increase in *CCA1*:*LUC* and *CAB2*:*LUC* expression immediately following lights on (Fig. 4), again implying an increase in light sensitivity in these plants relative to wild type. This suggests that the ability of the *elf4-1* mutant to anticipate dawn was attenuated, extending the possibility that entrainment of the oscillator is altered in *elf4-1*. In contrast, *ELF4-ox* correctly anticipated the coming lights on before photic signals were present (Fig. 4). We interpret this as a strong suggestion that, whereas *ELF4* is essential for normal entrainment to light, rhythmic accumulation of *ELF4* transcript is not.

The transcription of *CCR2* cycles, with a trough in the day and a peak in the night, is similar to the phase angle of *ELF4* (Fig. 6A). Under short days, only a marginal rhythm is seen for *CCR2*:*LUC* in *elf4-1*; however, a weak rhythm that apparently is able to anticipate dusk is seen in long photoperiod conditions (Fig. 4, E and F), suggesting that the slave oscillator of *CCR2* (Heintzen et al., 1997) still runs under these conditions even in the *elf4-1* mutant. Again, the same phase of the *CCR2* peak was seen in *ELF4-ox* plants compared to wild type (Fig. 4, E and F), reinforcing our earlier proposition that, whereas *ELF4* is necessary for correct entrainment of plants, rhythmic *ELF4* expression is not.

To further refine our understanding of clock resetting and *ELF4*'s role in this entrainment process, we measured the time taken by wild type, *elf4-1*, and *ELF4-ox* seedlings harboring *CCR2*:*LUC* to reentrain to a 12/12 LD cycle following the inversion of day and night (equivalent to jumping across 12 time zones instantaneously). The rapid change in light regime induces jet lag because the circadian clock is no longer in its correct orientation with respect to the environmental cycle. This protocol is similar to that used to define entrainment defects in *cca1* and *lhy* mutants (Kim et al., 2003; Fig. 2). Under this regime, the timing of peak *CCR2*:*LUC* activity, relative to the lights-out signal, was nearly restored in *elf4-1* within the first day (Fig. 2B). In contrast, the wild-type line did not display near-normal timing of the peak in *CCR2* expression until the second day. Thus, *elf4* resets faster than the wild type.

To understand the preliminary events that led to rapid clock resetting in *elf4-1* relative to wild type, we repeated the assay with the three genotypes expressing *LUC* under the control of *CCA1* or *LHY* promoters, respectively. This showed that the morning peak of *CCA1* and *LHY* in wild type occurred when plants expected dawn (e.g. after time 48 for CCA1 [Fig. 2C] or time 72 for *LHY* [Fig. 2D]), although this point was now in darkness because of the extended night. Wild-type plants exhibited little response to the lights on that occurred at subjective dusk (listed as time 60 for *CCA1* [Fig. 2C] and time 84 for *LHY* [Fig. 2D]), relative to the original entraining cycle (Fig. 2, C and D). These

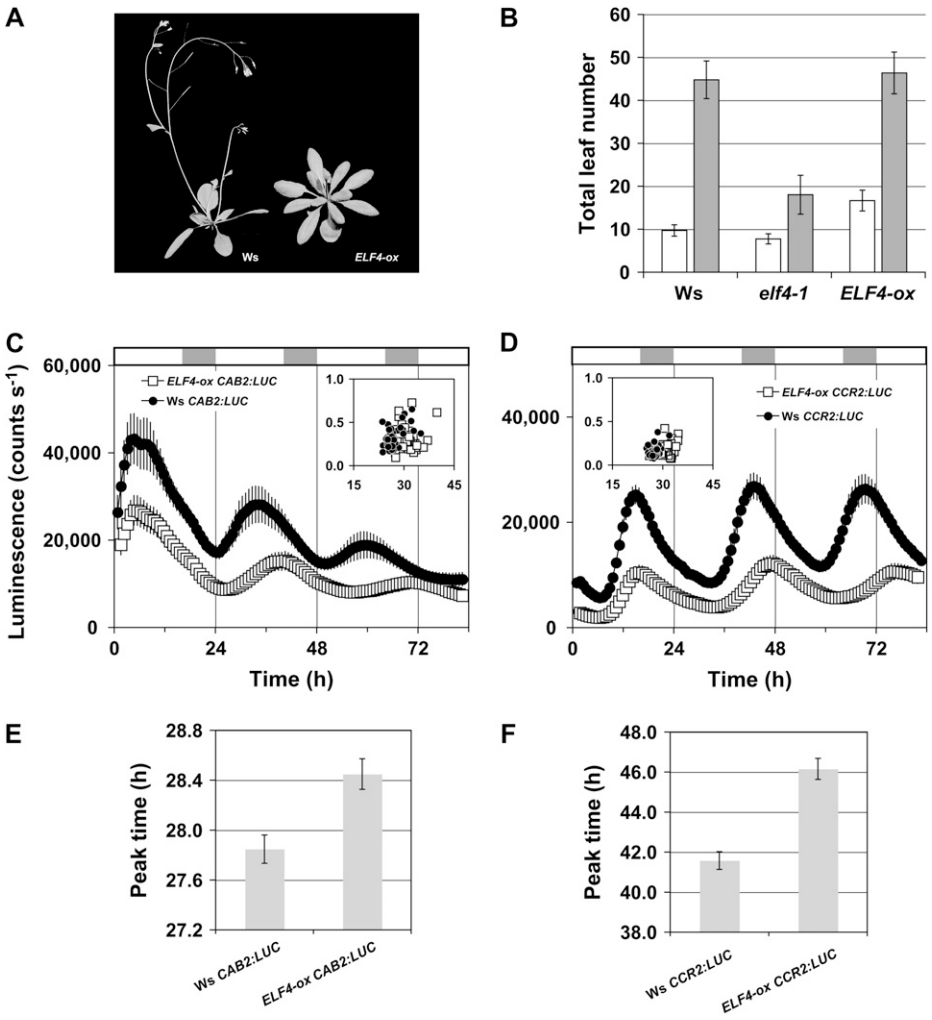

Figure 3. Dose-dependent effect of *ELF4*. A and B, *ELF4-ox* plants flower late under long days (white bars), but not short days (gray bars). C to F, *ELF4-ox* has long period and early phase under LL. *CAB2*:*LUC* (C and E) and *CCR2*:*LUC* (D and F). C and D, Gray bars indicate subjective night. Insets, RAE plots of luminescence rhythms (RAE versus period length). Each period estimate corresponds to one seedling. E and F, Peak time of *ELF4-ox CAB2*:*LUC* (E) and *CCR2*:*LUC* (F) in DD. Error bars represent SEM. All seedlings were entrained in 16/8 LD cycles. Time is ZT.

results can be explained by gated repression of light activation of these genes during the subjective night, similar to that shown for *CAB2* in wild-type seedlings (Fig. 1B). In contrast, the peak of *LUC* activity in *elf4-1* was much reduced after time 48, but the relative increase in gene induction in response to lights on at time 60 was much greater. This is consistent with the defective gating found in this mutant in which the gate for light responsiveness is open during subjective night. The light induction of *CCA1* and *LHY* in *elf4-1* is the likely cause of its rapid clock resetting.

ELF4-ox plants also exhibited accelerated clock resetting of *CCR2*:*LUC* relative to wild type. However, expression of *CCA1*:*LUC* and *LHY*:*LUC* in *ELF4-ox* matched that of wild type between time 36 and 72 for *CCA1* (Fig. 2C) and time 48 and 96 for *LHY* (Fig. 2D), implying that the resetting behavior here is not likely to be due to changes in the gating of light responsiveness. Instead, we suggest it may be due to the longer endogenous period allowing easier resetting via a single-phase delay.

Temperature Entrainment Defect in *elf4*

Temperature cycles can rescue rhythmicity of the *elf3* mutant, which acts to gate light input, in a subsequent interval of constant temperature (McWatters et al., 2000). *elf4-1* was therefore tested against wild-type plants for rescue of rhythmicity following exposure to warm-cold cycles. As before, entrainment to LD cycles failed to rescue subsequent free-running rhythmicity for all markers tested (*CAB2*, *CCA1*, or *CCR2*); all these reporters were arrhythmic in *elf4-1* populations grown under LL (Fig. 5, A, C, and E). In the absence of photoperiods, rhythmicity in *elf4* for all three reporters could be driven in warm-cold cycles (Fig. 5, B, D, and F). Thus, the *elf4* mutant can perceive ambient temperature cues. However, once the temperature cycle was discontinued, free-running rhythmicity was extremely weak beyond the first day in constant temperature for *CAB2*, *CCA1*, or *CCR2* in *elf4-1* (Fig. 5, B, D, and F). As expected, the control wild-type plants were robustly rhythmic under these conditions.

Timing of *ELF4* Action

ELF4 is required for robust rhythmicity and for a normal response to LD cycles. To aid the understanding of *ELF4*'s role in the circadian signaling network,

Table I. *Free-running period estimates*

RAE-weighted means and SEM for period length *ELF4-ox* lines and controls.

Line	Period	*n*
	h ± SEM	
ELF4-ox CAB2:LUC	31.60 ± 0.35	36
Ws *CAB2:LUC*	26.74 ± 0.33	38
ELF4-ox CCA1:LUC	31.17 ± 0.37	37
Ws *CCA1:LUC*	28.30 ± 0.31	44
ELF4-ox CCR2:LUC	30.08 ± 0.38	45
Ws *CCR2:LUC*	27.23 ± 0.18	45
ELF4-ox LHY:LUC	31.19 ± 0.40	46
Ws *LHY:LUC*	26.94 ± 0.29	48
ELF4-ox TOC1:LUC	30.40 ± 0.45	15
Ws *TOC1:LUC*	27.33 ± 0.44	24
Leaf movement		
ELF4-ox-2	25.87 ± 0.13	28
ELF4-ox-8	25.84 ± 0.21	29
ELF4-ox-11	25.60 ± 0.14	30
Ws	23.58 ± 0.10	42

molecular expression phenotypes of core clock genes were measured in various *ELF4* genotypic backgrounds. Luminescence rhythms were measured in wild-type plants expressing *ELF4:LUC* under LL after entrainment under LD cycles. Compared to the evening marker *CCR2*, *ELF4:LUC* generated a rhythm with peak expression in the middle of the night (Fig. 6A). We compare this to our analysis on the *ELF4* transcript under 12/12 LD photoperiods. There, we found peak expression at dusk (ZT12; Fig. 6G); we have previously shown that *ELF4* transcript levels are clock controlled and peak in the evening and that *ELF4* expression is affected by photoperiod (Doyle et al., 2002). Taken together, all of these results support an evening-to-night function of *ELF4* action and illustrate that the precise timing of the *ELF4* peak is influenced by the presence and/or duration of a photoperiod.

As expected, *ELF4:LUC* activity in *elf4* was arrhythmic (Fig. 6B), as was that of *CCR2:LUC* expression, in agreement with our previous reports. Rhythmicity in the *elf4-1* mutant could be rescued by restoring *ELF4* expression with the *ELF4:ELF4-LUC* construct (Fig. 6B). Like plants constitutively overexpressing *ELF4*, these plants had a long-period phenotype. Thus, ELF4 regulation appears to be primarily transcriptional and ELF4 activity is potentially dose dependent even under the control of its own promoter.

The *elf4* mutant phenotype includes low transcription of the morning clock gene *CCA1* (we confirmed our previous *LUC* data regarding *CCA1* expression [Doyle et al., 2002] by direct analysis of its RNA [Supplemental Fig. S2A]). In contrast, *CCA1:LUC* rhythms were increased in amplitude and had a long period in plants that constitutively overexpressed *ELF4* (Table I; Fig. 6C; Supplemental Fig. S3A). It is thus likely that *ELF4* is a limiting factor in *CCA1* induction. Also, in the *elf4-1* mutant, *LHY:LUC* was repressed to a very low level and was arrhythmic (Fig. 6D), as was *LHY* transcript expression (Supplemental Fig. S2B), similar to the findings for *CCA1* and *LHY* expression reported previously (Doyle et al., 2002; Kikis et al., 2005). Again, *ELF4-ox* plants displayed long-period *LHY:LUC* rhythms (Table I; Fig. 6D; Supplemental Fig. S3B). Thus, *ELF4* is likely to control activation of both morning acting clock genes, *CCA1* and *LHY*.

The current model of the *CCA1/LHY-TOC1* loop (Alabadi et al., 2001; Locke et al., 2005) predicts an increase in *TOC1* expression wherever there is given low *CCA1* and *LHY* expression. Expression of *TOC1:LUC* in the null *elf4-1* allele followed this prediction, being expressed arrhythmically and at a higher level in *elf4-1* than wild-type seedlings free-running under LL conditions (Fig. 6E; Supplemental Fig. S3C). This finding was confirmed by examining *TOC1* transcript expression in *elf4-1* directly by real-time reverse transcription-PCR (Supplemental Fig. S2C); *TOC1* expression was high and became arrhythmic within 24 h of the transfer to LL. In addition, we found that *ELF4* transcription in the *toc1* mutant was rhythmic, with an early phased peak (Fig. 6G), similar to the phase of *ELF4* expression in the *cca1 lhy* double mutant (Kikis et al., 2005). Rhythmicity of *TOC1:LUC* was maintained in lines overexpressing *ELF4*, but there was reduced amplitude; we observed wild-type levels of *TOC1* transcript in *ELF4-ox* (data not shown). As anticipated, *TOC1:LUC* rhythms displayed a long-period response in *ELF4-ox* (Table I; Fig. 6F; Supplemental Fig. S3D). Taken together, these last results suggest strongly that *ELF4* is necessary for the feedback loop controlling rhythmicity of *CCA1*, *LHY*, and *TOC1*, where it acts at night to promote *CCA1/LHY* expression and thus indirectly represses *TOC1*.

DISCUSSION

Our data illustrate that *elf4-1* plants have a range of deficiencies in their circadian responses to light, photoperiod, and temperature. Importantly, such plants do not display sustained rhythmicity in the absence of environmental signals. Misexpression studies of *ELF4* further confirm an important clock function for this gene. Analysis of gene expression of key components of the plant clock (*CCA1*, *LHY*, and *TOC1*) and targeted assays to define the abrogated rhythm in *elf4-1* revealed that the central circadian feedback loop in *elf4* was locked into the evening phase. However, constitutive overexpression of *ELF4* does not produce arrhythmia, but acts to delay the clock, causing a long-period phenotype seen across a range of assays. Plants overexpressing *ELF4* exhibited robust rhythms of clock gene expression and these lines were able to respond to photoperiods, for example, flowering earlier in long days than short days. These results showed that *ELF4* is essential for free-running circadian rhythms.

Here, we have presented evidence that expression of the various clock outputs is strongly affected by the LD zeitgeber in the *elf4-1* mutant (Figs. 2 and 4). *elf4-1*

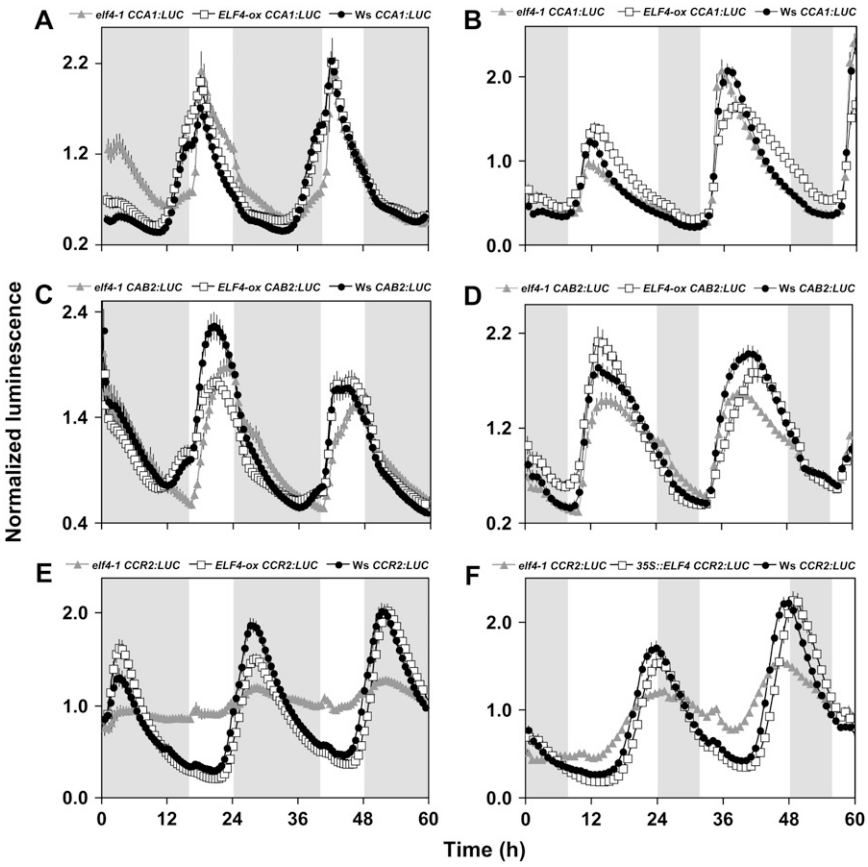

Figure 4. Morning gene expression (*CCA1*:*LUC*, *CAB2*:*LUC*) is less affected than expression of an evening-specific gene (*CCR2*:*LUC*). Luminescence profiles of *elf4-1* and *ELF4-ox* kept under entraining conditions, 8/16 LD short day (left) and 16/8 LD long day (right). A and B, *CCA1*:*LUC*. C and D, *CAB2*:*LUC*. E and F, *CCR2*:*LUC*. Gray blocks indicate night time. Error bars represent SEM.

plants were found to show more rapid reentrainment following a change in the zeitgeber phase (Fig. 2, B–D), which indicates that the clock is reset more rapidly in these mutants than in the wild type. This is probably due to *ELF4*'s role of gating light input to the clock. The gate in *elf4-1* plants never fully closes (Fig. 1B); hence, these plants are more sensitive to photic cues due to increased activity of the light-signaling pathway. Increased light sensitivity is also seen in the pattern of *CAB2*:*LUC* and *CCA1*:*LUC* expression in *elf4* mutants (Fig. 4). In the absence of a zeitgeber, *elf4-1* does not show robust free-running rhythms in any of the various hands of the clock (Figs. 5 and 6). Regardless of how the clock is assayed, the *elf4-1* mutant is weakly rhythmic or arrhythmic under LL and DD, regardless of the previous entrainment protocol. We thus conclude that *ELF4* is required for entrainment.

Most importantly, the putative clock components *CCA1*, *LHY*, and *TOC1* are virtually arrhythmic after the first 24 h in LL in an *elf4-1* background, implying that this feedback loop cannot continue to cycle in the absence of *ELF4*. We have shown that *CCA1* and *LHY* levels are both low in the *elf4-1* mutant, whereas *TOC1* is high—strong circumstantial evidence that *ELF4* acts to promote the former while repressing the latter (Fig. 6; Supplemental Fig. S2). This evidence leads to the conclusion that *ELF4* is essential for correct clock function in Arabidopsis and that, in the absence of *ELF4*, the clock will stop after a single cycle. *ELF4* transcription is rhythmic, with a peak during the early night, coinciding with the point at which the clock arrests in *elf4-1*, implying that *ELF4* acts at this point of the 24-h cycle (Fig. 2A). A recent study by Quail and colleagues reported that *TOC1* expression was unchanged in another *elf4* mutant allele (*elf4-101*, a T-DNA insertion in the Columbia background; Kikis et al., 2005). A difference between *elf4* alleles or genetic backgrounds might account for the discrepancy between their study and ours. However, a more plausible explanation is that experimental protocols differed widely. Our results show that the *elf4-1* mutant has residual rhythmicity for 1 d following entrainment. In the earlier study, dark-grown seedlings were assayed for *TOC1* levels immediately after 24 h under constant red light (Kikis et al., 2005).

The near loss of circadian function in *elf4* differs from all previously described recessive circadian mutants of Arabidopsis because *elf4* mutants become quickly arrhythmic when transferred to all types of constant conditions. Other mutants, for example, *elf3* and *lux*, are capable of maintaining rhythmicity in certain unchanging constant conditions, such as DD (Hicks et al., 1996, 2001; Covington et al., 2001; Hazen et al., 2005). Moreover, elimination of any one of the three putative central clock components, *CCA1*, *LHY*, and *TOC1*, does not stop the clock, but merely confers

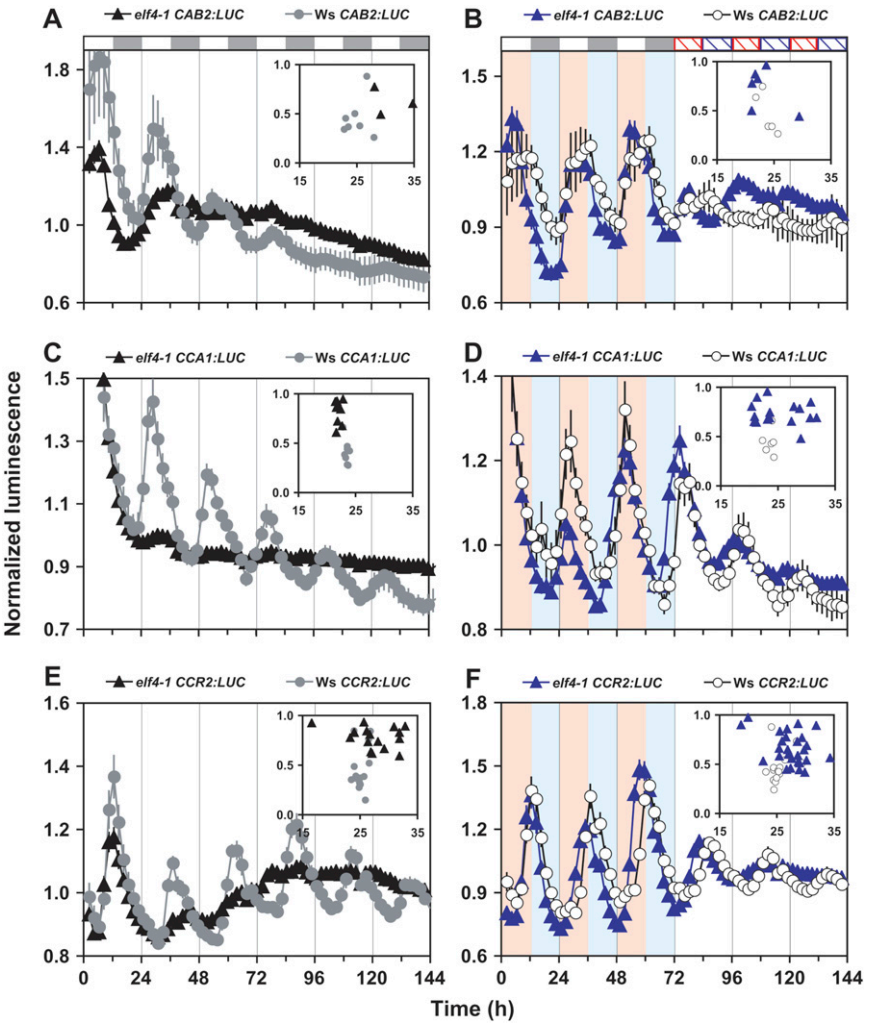

Figure 5. Temperature entrainment defects in *elf4-1*. A, C, and E, Seedlings were entrained to LD cycles and then transferred to LL. B, D, and F, One set of LD-entrained plants was subsequently given temperature cycles for 3 d (12:12 warm/cool [WC]) and then released into LL and constant temperature for 3 d. A and B, *CAB2:LUC*. C and D, *CCA1:LUC*. E and F, *CCR2:LUC*. White bars indicate free run under LL. A, C, and E, Gray bars indicate subjective night. B, D, and F, Red blocks indicate 24°C (daytime). Blue blocks indicate 18°C (nighttime). Red hatched bars indicate subjective warm day. Blue hatched bars indicate subjective cold night. Time is light ZT. Insets, RAE plots (RAE versus period). Each period estimate is an RAE-weighted mean of a group of seedlings. Hours 12 to 84 and 84 to 152 analyzed for free run under LL after LD and WC entrainment, respectively. Error bars represent SEM.

a short period upon the output rhythms (Somers et al., 1998; Alabadi et al., 2002). *ELF4* transcription remains rhythmic in both the *toc1* mutant (Fig. 6G) and the *cca1 lhy* double mutant (Kikis et al., 2005); in each case, *ELF4* expression has an early phase. Thus, the relationship between *ELF4* and other clock genes appears asymmetric: *ELF4* is required for rhythmicity of other clock-associated genes, but they are not required for *ELF4* rhythms to exist, although they do drive the correct phase of *ELF4*.

We have shown that, although the *CCA1/LHY-TOC1* feedback loop is stalled in the evening phase in *elf4-1*, the clock has full oscillatory function in *ELF4-ox*, which shows robust rhythmicity of gene expression (Table I; Figs. 5 and 6), and this line is able to distinguish between long and short days for the purpose of controlling flowering time. However, the long-period phenotype and later flowering under long days of *ELF4-ox* plants highlights the notion that the level of *ELF4* expression calibrates circadian period. We have previously observed that *ELF4* levels are extremely low and lose rhythmicity in wild-type plants grown in extended darkness (Doyle et al., 2002), yet the *CCA1/LHY-TOC1* feedback loop continues in wild-type plants under these conditions. These two observations lead us to suggest that, although transcription of *ELF4* is normally rhythmic (due to exposure to natural LD and temperature cycles), and the presence of *ELF4* is sufficient to drive this loop, rhythmic *ELF4* transcription is not necessary for the clock to sustain oscillatory function.

A previous report on an *elf4* mutant allele that demonstrated arrhythmicity of the *CCA1/LHY-TOC1* feedback loop in dark-grown seedlings indicated that *ELF4* was required for light activation of this loop (Kikis et al., 2005). We show here that *ELF4* is required to sustain this loop under LL (Figs. 1, A and B, and 6). Taken together, these observations indicate that *ELF4* is necessary to start the clock, sustain it under constant conditions, and enable it to entrain to a zeitgeber. These conclusions considerably extend the earlier model that placed *ELF4* in a light input loop with *CCA1* and *LHY* (Kikis et al., 2005).

We suggest that *ELF4* functions to convert an hourglass into a clock. Without *ELF4*, the *CCA1/LHY-TOC1* feedback loop can be turned over by an environmental

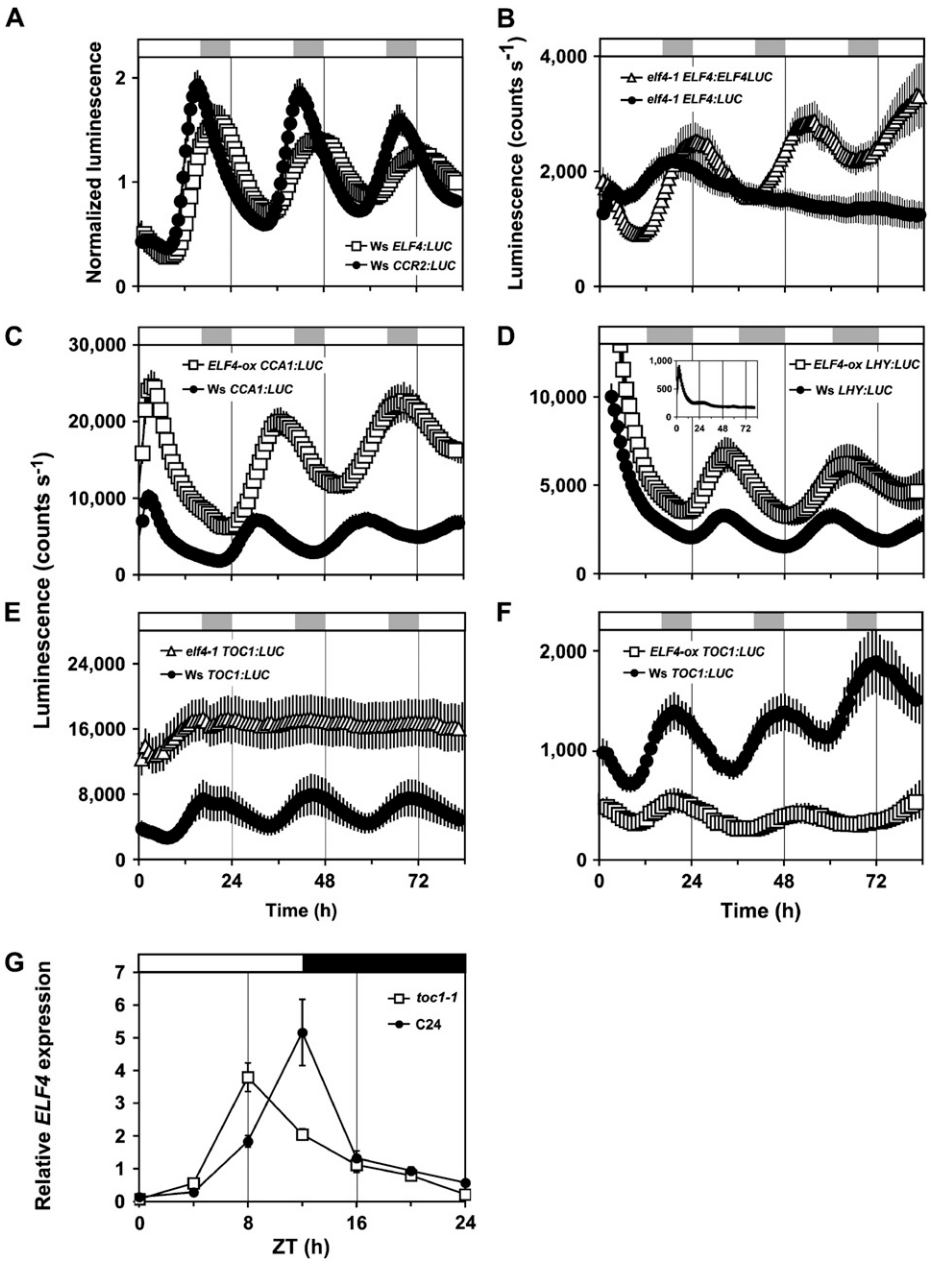

Figure 6. *ELF4* is expressed in the night and influences the expression level of *CCA1* and *TOC1*. A, *ELF4*:*LUC* luminescence activity compared to *CCR2*:*LUC* in wild type. B, Luminescence of *ELF4*:*ELF4LUC* and *ELF4*:*LUC* in the *elf4-1* mutant. C, Long period and high amplitude of *CCA1*:*LUC* in *ELF4-ox* under LL. D, Long period of *LHY*:*LUC* in *ELF4-ox*. Inset, *LHY*:*LUC* expression in *elf4-1* mutant; note that in *elf4-1* the LUC levels are arrhythmic and more than 10-fold lower than the wild type. E, *TOC1*:*LUC* expression is high and arrhythmic in *elf4-1*. F, *ELF4-ox* displays low *TOC1*:*LUC* expression, which is robustly rhythmic. Gray bars indicate subjective day. Time is ZT. Error bars represent SEM. All LUC seedlings were entrained in 16/8 LD cycles. G, *ELF4* expression is rhythmic in the *toc1-1* mutant. Seedlings were entrained in 12/12 LD cycles. *ELF4* level is normalized to *β-TUBULIN4* at each time point. Mean values are plotted; error bars represent SDS. The experiment was replicated twice.

cycle of light and dark, but stops depending on the discontinuation of the environmental rhythm. The closest functional analog to *ELF4* may be the *FREQUENCY* (*FRQ*) locus of *Neurospora crassa*. In the absence of *FRQ*, *N. crassa* rhythms are of low amplitude, variable length, and not temperature compensated (Merrow et al., 1999, 2006). Previous reports, our own included, have placed *ELF4* as part of a light input pathway to the clock. Current data reported allow us to revise this interpretation and state that, because *ELF4* is essential for at least two critical clock properties, sustainability and entrainment, it should be considered a core clock component. Assignment of function to *FRQ* remains a controversial issue (Merrow et al., 1999; Pregueiro et al., 2005; Ruoff et al., 2005; de Paula et al., 2006; Lakin-Thomas, 2006; Schafmeier et al., 2006); whether it becomes so with *ELF4* remains to be seen.

MATERIALS AND METHODS

Plant Material and Transgenics

Arabidopsis (*Arabidopsis thaliana*) ecotype Wassilewskija (Ws-2), *elf4-1*, and the *LUC* lines *CAB2*:*LUC*$^+$ (6B insertion), *CCA1*:*LUC*, and *CCR2*:*LUC* are described (McWatters et al., 2000; Doyle et al., 2002). The *toc1-1* mutant is in the C24 background and has been described previously (Somers et al., 1998). For the vector to generate lines overexpressing *ELF4*, first the 35S promoter and Nos terminator were subcloned from pBI121 into pZIP221B as a

*Hin*dIII-*Eco*RI fragment. The *ELF4* coding region was amplified against genomic DNA by PCR with the primers ELF435S-L, 5′-AAAAGATCTCC-GGTCCAACTAAGAAGAAACAAT-3′ and ELF435S-R, 5′-AAAAGATCTC-GACTTTGACGAAAATCAAAAAG-3′ and this fragment was subcloned between the 35S promoter and the terminator as a *Bam*HI fragment. This construct was used to generate multiple homozygous lines that overexpressed *ELF4* (*ELF4-ox*). All tested lines behaved similarly in all assays. Wild-type Ws lines harboring the *CAB2*:*LUC*$^+$, *CCA1*:*LUC*, or *CCR2*:*LUC* transgene were crossed into both the *elf4-1* mutant and the *ELF4-ox* line termed *ELF4-ox-11*; in each case, double homozygous lines were identified in the F_2 generation and bulked. We report experiments using the F_3 generation of these lines. Gating experiments utilized the *CAB2*:*LUC* transgenic line (i.e. the 2CA/C insertion present in accession C24; Millar et al., 1995) introgressed into Ws and *elf4-1*.

ELF4:*LUC* was constructed by subcloning approximately a 1.5-kb fragment from the bacterial artificial chromosome clone T28M21 as a *Xba*I-*Nco*I fragment into pZIP221B. This fragment is between 1.7 kb and 260 bp upstream of the translational start site. The *LUC*$^+$ gene from pLUC$^+$ (Promega) was subcloned into this resultant vector as a *Nco*I-*Xba*I fragment (pELF4-incomplete:LUC$^+$). A fragment was PCR amplified using an arbitrary upstream primer and the primer 5′-AACCATGGTCTCGCCGTTCCTCTTCATAA-3′. This PCR product was digested with *Nco*I and the fragment intersected into the *Nco*I site of pELF4-incomplete:LUC$^+$, resulting in the completed transcriptional fusion pELF4P:LUC$^+$. Similarly, an upstream primer and 5′-AAACCATGGCTCT-AGTTCCGGCAGCACCAC-3′ was used to generate a PCR that was subcloned as an *Nco*I fragment into pELF4-incomplete:LUC to generate the translational fusion construct pELF4P:ELF4-LUC$^+$.

To generate a vector for *TOC1*:*LUC*, PCR against Ws genomic DNA using 5′-TCGCTCTAGACTTCTCTGAGGAATTTCATCAAAC-3′ and 5′-ACT-AGGATCCGATCAGATTAACAACTAAACCCACA-3′ generated a 2,068-bp fragment that was subcloned into a LUC vector as a *Xba*I-*Bam*HI insert and, for *LHY*:*LUC*, a similar PCR with 5′-TGCGGTCGACTGTTTCAAATAACTGT-TATGTCCTA-3′ and 5′-GGAAGGATCCAACAGGACCGGTGCAGCAT-3′ generated a 1,812-bp fragment that was subcloned as a *Sal*I-*Bam*HI insert. These constructs were used to transform wild-type Ws or *elf4-1*, as described in the text, by the floral-dip method (Clough and Bent, 1998). Experiments comparing *TOC1*:*LUC* expression in the wild type and *elf4-1* represent the averages of 24 lines each from six independent transgenics; all lines behaved similarly. Representative transgenic lines of *TOC1*:*LUC* and *LHY*:*LUC* were used in crosses to *ELF4ox-11*; the same *LHY*:*LUC* line was similarly introduced to *elf4-1* for experimentation.

Growth Conditions

For hypocotyl length measurements, seeds were surface sterilized and plated on 2.2 g/L Murashige and Skoog medium without Suc or vitamins (0.5× Murashige and Skoog) with 2.5 mM 2-(*N*-mopholino)ethanesulfonic acid (pH 5.7) and 8 g/L agar. Plates were stored in the dark for 3 to 4 d at 4°C, placed at 22°C in the darkness for 1 d, and irradiated with light for 6 d, as described (Davis et al., 2001). The light sources were as described in Hall et al. (2003). For circadian and real-time PCR experiments, seeds were similarly treated, except that they were plated on 1× Murashige and Skoog medium (4.4 g/L) with 3% Suc and 1% agar before being stratified at 4°C for 48 h. The seeds were then transferred to growth chambers programmed for appropriate light and temperature regimes for 7 d before the start of an experiment. Unless otherwise stated in the text, the fluence rate of white light was 65 μmol m^{-2} s^{-1} and plants were grown at a constant temperature of 22°C.

Rhythm Data Analysis

Luminescence levels were quantified on either a low-light imaging system or an adapted microtiter plate-reading scintillation counter and analyzed essentially as described (McWatters et al., 2000; Thain et al., 2000), using the software package MetaMorph (Universal Imaging Corp.), and the macro suites I&A, TopTempII, and Biological Rhythms Analysis Software System (Southern and Millar, 2005; available at http://www.amillar.org) and fast Fourier transform-nonlinear least squares (Plautz et al., 1997). Sustainability (precision) of rhythms was derived from measurements of the relative amplitude of error (RAE) as a method that has previously been reported (Allen et al., 2006; Izumo et al., 2006). Where appropriate, data were normalized. Here, normalization was plotted as the quotient of the absolute data point over the mean of the entire dataset, as a method in identical fashion to Hall et al. (2003). This allows the data to be qualitatively compared for each genotype while plotting on the same axes and preserving the waveforms.

Real-Time PCR

Replicated samples of *elf4-1*, *toc1-1*, and the wild-type controls Ws-2 and C24 seedlings were collected and immediately frozen in liquid nitrogen, starting at dawn on day 8. *toc1-1* and C24 seedlings were collected during a 12/12 LD cycle; *elf4-1* and Ws-2 seedlings under LL following the discontinuation of such a cycle. RNA was extracted (Qiagen RNeasy kit) using an additional DNAse treatment step (Qiagen) as per the manufacturer's instructions. cDNA was synthesized (ABI TaqMan) and real-time PCR carried out in triplicate in an ABI Prism 3700 using SYBR Green master mix (ABI) and gene-specific primers (*ELF4*-F, 5′-CGACAATCACCAATCGAGAATG-3′, *ELF4*-R, 5′-AATGTTTCCGTTGAGTTCTTGAATC-3′; *TOC1*-F, 5′-ATCTTCGCAGAA-TCCCTGTGATA-3′, *TOC1*-R, 5′-GCACCTAGCTTCAAGCACTTTACA-3′; *CCA1*-F, 5′-TCTGTGTCTGACGAGGGTCGAATT-3′, *CCA1*-R, ACTTTGCGG-CAATACCTCTCTGG-3′; *LHY*-F, 5′-CAACAGCAACAACAATGCAACTAC-3′, *LHY*-R, 5′-AGAGAGCCTGAAACGCTATACGA-3′; *β-TUBULIN4*-F, 5′-TTT-CCGTACCCTCAAGCTCG-3′, *β-TUBULIN4*-R, 5′-TGAGATGGTTAAGATC-ACCAAAGG-3′). Levels of circadian gene and the control gene *β-TUBULIN4* in each sample were calculated using the standard curve method (Applied Biosystems, User Bulletin no. 2, 2001 update). Circadian gene expression was then normalized using contemporaneous *β-TUBULIN4* expression from the same sample.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. A, Expression of *ELF4* RNA is increased in *ELF4-ox* plants. B, Representative leaf movement traces of *ELF4-ox* and Ws imaged under LL.

Supplemental Figure S2. RNA expression of putative central clock genes in *elf4-1* and Ws.

Supplemental Figure S3. Period estimates of *CCA1*:*LUC* (A) and *LHY*:*LUC* (B) in *ELF4-ox*. Period analysis of *TOC1*:*LUC* in *elf4-1* (C) and *ELF4-ox* (D).

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